Chapter 2

Expression and Purification of Recombinant Cyclins and CDKs for Activity Evaluation

Edurne Gallastegui and Oriol Bachs

Abstract

Cyclin-dependent kinases (Cdks) belong to a family of key regulators of cell division cycle and transcription. Their activity is mainly regulated by association with regulatory subunits named cyclins but their activities are also regulated by phosphorylation, acetylation, and the association with specific inhibitory proteins (CKIs). The activity of different Cdks is deregulated in many different type of tumors, and thus, Cdks are considered targets for antitumoral therapy. For large screenings of inhibitors the use of purified recombinant Cdks and cyclins is recommended. We report here the current methods to determine their in vitro activity for large screenings of inhibitors.

Key words Cdk1, Cdk2, Cyclin A, Cyclin B, Kinase assay

1 Introduction

Regarding cell cycle regulation, the most important cyclins are the D-type cyclins that include three members (cyclins D1, D2, and D3), cyclin E, cyclin A, and cyclin B [1]. During cell cycle progression different Cdk–cyclin complexes are generated each one operating at specific moments along the cell cycle [2]. Specifically, Cdk4/6–cyclins D complexes are activated during the G_1 phase of the cell cycle and they are responsible of phosphorylating members of the pocked proteins family (pRb, p107, and p130) that are in transcriptional repressor complexes [3]. Subsequent phosphorylation of these complexes, by Cdk2–cyclin E, disrupts them inducing the transcription of genes encoding proteins necessary for DNA replication and mitosis [4]. Cdk2–cyclin E complexes are also involved in the triggering of DNA replication. Cdk2–cyclin A are subsequently activated and are necessary for the progression of DNA replication during the S phase of the cell cycle. Finally, Cdk1–cyclin A and Cdk1–cyclin B participate in the triggering and progression of mitosis [5].

Mar Orzáez et al. (eds.), *Cyclin-Dependent Kinase (CDK) Inhibitors: Methods and Protocols*, Methods in Molecular Biology, vol. 1336, DOI 10.1007/978-1-4939-2926-9_2,

The activities of different Cdks are deregulated in many different types of tumors, so they are considered relevant targets for antitumoral therapy [6]. To identify specific Cdk inhibitors, protocols to determine their activity have been developed. The in vitro Cdk activity is mainly determined by two general protocols. The first is by using Cdk–cyclin complexes obtained by immunoprecipitation (IP) of cell samples with antibodies against specific Cdks or cyclins as a source of the enzymes (Chapter 1). The second is by using purified recombinant Cdks and cyclins. When performing kinase assays using purified recombinant proteins, a more defined composition of the complexes can be achieved. When proteins are expressed in and purified from bacteria, the post-translational modifications (phosphorylation and acetylation) of Cdks are not produced and additionally no CKIs are associated. However, because the activating phosphorylation at T160 is not produced, the activity of the complexes that need this phosphorylation for full activity only would display a reduced activity. This problem can be solved by in vitro phosphorylating this T160 residue by incubation of the purified Cdk with the CAK enzyme previously to mix the Cdk with the selected cyclin. Finally, in vitro association of specific Cdks with their cyclin partners in some cases might be highly inefficient. For instance, the association of Cdk4 with D-type cyclins is one of these cases. The CKIs p21 and p27 can work as adaptor proteins that stimulate the interaction between both subunits [7]. However, because p21 and p27 also work as inhibitors of these complexes the in vitro activity of these complexes using purified recombinant proteins is difficult.

We describe here the protocols to determine Cdk1 and Cdk2 activities by using recombinant proteins expressed in and purified from bacteria. These protocols are recommended when the aim is to screen high amount of products on trying to identify specific inhibitors of these complexes. To analyze the activity of other Cdk–cyclin complexes, specific modifications of the protocols have to be done.

2 Materials

2.1 Expression and Purification of Recombinant Cyclins and Cdks for Determination of Kinase Activity

1. *LB* (*Luria–Bertani*) *medium*: 1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl (Autoclave before use).
2. *Antibiotics*: ampicillin (final concentration: 50 μg/ml) and chloramphenicol (final concentration: 20 μg/ml).
3. *Bacteria strain*: BL21 (DE3).
4. *IPTG*.
5. *Lysis buffer NETN+Inhibitors*: 20 mM Tris–HCl pH 8, 100 mM NaCl, 1 mM EDTA, 0.5 % NP-40, 0.5 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 0.1 mM Na_3VO_4.

6. *Glutathione-Sepharose-4B resin*.
7. *Elution buffer*: 50 mM Tris–HCl pH 9.6, 120 mM NaCl, 20 mM glutathione reduced.
8. *1× PBS*: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4.
9. *Bradford reagent*.

3 Methods

3.1 Protein Expression

Cdk and cyclin cDNA must be cloned in a pGEX plasmid. This kind of plasmids is used to express high amounts of proteins fused to glutathione S-transferase (GST).

1. Transform pGEX plasmids containing cDNAs of interest in BL21 (DE3) and grow in LB medium with ampicillin and chloramphenicol at 37 °C with shaking to mid-log phase (OD_{600nm} 0.8).
2. Induce the expression by the addition of isopropyl 1-thio-d-galactopyranoside (IPTG) at a final concentration of 0.5 mM and incubate the culture for 4 h with shaking at room temperature.
3. To test the induction, run 30 μl of non-induced and induced bacteria culture in a polyacrylamide gel and stain with Coomassie Blue.

3.2 Protein Purification

1. Harvest the bacteria by centrifugation at 4000 × *g* at 4 °C for 10 min.
2. Suspend the pellet in NENT buffer + Inhibitors (*see* **Note 1**).
3. Froze the bacterial cells at −80 °C and thaw on ice three times.
4. Lyse the cells by sonication four times for 10 s.
5. Perform a centrifugation at 24,000 × *g* at 4 °C for 15 min.
6. Do the purification using affinity chromatography by incubating the supernatant with Glutathione-Sepharose-4B resin in a column. For every 5 mg of protein 1 ml of Glutathione-Sepharose-4B has to be used (*see* **Note 2**).
7. Incubate the mix with shaking at 4 °C for 1 h.
8. Wash three times with NENT (final volume of 100 ml).
9. At this point, GST from recombinant proteins can be removed if needed but to perform the kinase assay this step is not necessary (*see* **Note 3**).
10. Add 5 ml of elution buffer and incubate with shaking at 4 °C for 10 min.

11. Protein is obtained after centrifuging at 2000×*g* at 4 °C for 5 min and keeping the supernatant.
12. Dialyze the purified recombinant proteins against PBS and calculate the concentration by Bradford assay.

3.3 Kinase Assay

1. Mix 400 nM of Cdk protein and the same concentration of cyclin protein in a tube and incubate for 10 min on ice (as many tubes as reactions we want to perform are prepared at this step). If the effect of some inhibitor wants to be studied, it has to be added to the mix at this point.
2. Kinase assay and polyacrylamide gel electrophoresis are performed as previously described (*see* Chapter 1), depending on the Cdk–cyclin activity to be determined. For Cdk2, Cdk4/6, and Cdk1 activities *see* Subheadings 3.1, 3.2, and 3.3, respectively. For material related to Cdk2, Cdk4/6, and Cdk1 kinase assays *see* Subheadings 2.1, and Subheadings 2.2, and 2.3, from Chapter 1 (E. Gallastegui and O. Bachs).

4 Notes

1. The NETN buffer can be prepared and stored at 4 °C, but the inhibitors must be added at the beginning of the protocol (0.5 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 0.1 mM Na_3VO_4).
2. 5 mg of protein is equal to a starting volume of bacterial culture of 0.5–1 l, approximately.
3. If desired, GST can be removed from the GST fusion proteins by digestion with thrombin protease.

References

1. Malumbres M, Barbacid M (2005) Mammalian cyclin-dependent kinases. Trends Biochem Sci 30:630–641
2. Morgan DO (1997) Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 13:261–291
3. Malumbres M (2014) Cyclin-dependent kinases. Genome Biol 15:122–131
4. Rubin SM (2013) Deciphering the retinoblastoma protein phosphorylation code. Trends Biochem Sci 38:12–19
5. Lindqvist A, Rodríguez-Bravo V, Medema RH (2009) The decision to enter mitosis: feedback and redundancy in the mitotic entry network. J Cell Biol 185:193–202
6. Asghar U, Witkiewicz AK, Turner NC, Knudsen ES (2015) The history and future of targeting cyclin-dependent kinases in cancer therapy. Nat Rev Drug Discov 14:130–146
7. LaBaer J, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A, Harlow E (1997) New functional activities for the p21 family of CDK inhibitors. Genes Dev 11:847–862